An Electron Paramagnetic Resonance (EPR) spectroscopy study on the γ-irradiation sterilization of the pharmaceutical excipient L-histidine: regeneration of the radicals in solution

C. Vallotto,[a] H. E. Williams,[b] D. M. Murphy,[c] Z. J. Ayres,[d] R. Edge,[e] M. E. Newton[a] and C. J. Wedge[a,f,*]

[a] Department of Physics, University of Warwick, Coventry, CV4 7AL, UK.

[b] Product Development, AstraZeneca, Silk Road Business Park, Macclesfield, SK10 2NA, UK.

[c] School of Chemistry, Cardiff University, Main Building, Park Place, Cardiff, CF10 3AT, UK.

[d] Department of Chemistry, University of Warwick, Coventry, CV4 7AL, UK.

[e] Dalton Cumbrian Facility, The University of Manchester, Westlakes Science & Technology Park, Moor Row, Cumbria CA24 3HA, UK.

[f] Present address: Department of Chemical Sciences, University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, UK.

*Corresponding Author. Email address: c.wedge@hud.ac.uk Tel: +44 1484 47 1614

ABSTRACT

The effects of γ-radiation sterilization on the parenteral excipient L-histidine were analysed by means of EPR spectroscopy. The irradiation process was found to induce the formation of a deamination radical which was persistent in the solid state. The nature and reactivity of the radicals following dissolution in water was evaluated using spin-trapping EPR experiments. The deamination radical was found to regenerate in solution in the presence of trace metals, potentially leading to radical induced degradation reactions occurring up to an hour after the dissolution process. Understanding this process is significant for the improved design of parental pharmaceutical formulations in which unwanted radical reactions after γ-radiation sterilization could lead to degradation of active ingredients.

Keywords

Electron Paramagnetic Resonance (EPR)

Spin-trapping

Sterilization

Irradiation

Dissolution

Fenton reaction

Chemical compounds studied in this article

L-histidine (PubChem CID: 6274); 2-Methyl-2-nitrosopropane (PubChem CID: 23272);

1. Introduction

Radiation sterilization of pharmaceuticals has been studied for over 50 years as a means to allow sterilization of heat sensitive materials (Gopal, 1978). When applicable, this terminal-sterilization process is preferable to other methods such as gas sterilization, which is difficult to control, or aseptic processing, which is a complex and costly procedure (Food and Drug Administration, 2011; Hasanain et al. 2014). Radiation sterilization is usually carried out with a γ-source primarily due to the high penetrating power, speed, reliability and facile control of the process (Abuhanoğlu and Özer, 2010). This irradiation process may produce free radicals (Symons, 1995); these extremely reactive species frequently promote a number of different chemical reactions, which are difficult to predict beforehand (Schulman and Achey, 2007). It is therefore necessary to assess the stability of each irradiated sample and thereby characterize the degradants formed as a result of the sterilization process (Ambroż et al., 2000; Gibella et al., 2000; Hasanain et al. 2014; Jacobs, 1995, 1985).

Excipients are substances other than the pharmacologically active drugs or prodrugs which are included in the manufacturing process or are contained in a finished pharmaceutical product dosage form (Pikal and Costantino, 2004). These excipients improve the properties of the drug, either by enhancing the therapeutic effect of the Active Pharmaceutical Ingredients (APIs) or by facilitating the manufacturing process (García-Arieta, 2014), and are typically the major components in a pharmaceutical product. Not only could direct degradation of the APIs diminish the action of the product, but degradation of excipients can also affect the efficacy of the drug either by altering its chemico-physical properties or by reacting with the APIs. It is therefore crucial to assess the stability of such components after they undergo industrial processes which could affect their stability.

In this work we focus on the amino acid L-histidine (hereafter labelled L-his), an excipient typically used in parenteral formulations as a buffering agent and a stabilizer for subcutaneous, intramuscular and peritoneal injections (Kaisheva et al., 2003; Kamerzell et al., 2011). The effects of γ- and X-irradiation on L-his has been studied by means of Electron Paramagnetic Resonance (EPR) spectroscopy, which detects specifically paramagnetic species, such as free radicals, with the unaltered L-his or non-radical degradation products remaining EPR silent (Mangion et al., 2016). The identity of the main radical species generated by irradiation was confirmed by EPR analysis of both the L-his powder and the single crystal. The irradiation products of numerous amino acids have been investigated previously by EPR in the solid state (Aydin, 2010; Dicle et al., 2015; Karabulut and Yıldırım, 2015), but here the reactivities of the radicals following dissolution were also evaluated by means of spin-trapping EPR experiments (Davies, 2016). While studies involving the spin trapping of radicals formed in the solid state have been previously reported for several organic compounds, including amino acids (Kuwabara et al., 1981; Lagercrantz and Forschult, 1968; Makino and Riesz, 1982; Minegishi et al., 1980; Talbi et al., 2004) we are not aware of previous reports of the regeneration and trapping of amino acid radicals in solution upon addition of the trapping agent many minutes after dissolution of an irradiated powder.

2. Material and methods

2.1. γ-irradiation of powder

L-his free base was purchased from Sigma Aldrich and irradiated in the supplied powder form. Samples were sealed in glass vials and γ-irradiated at room temperature (r.t.) at the Dalton Cumbrian Facility (UK) using a dose rate of approximately 2.3 kGy/h to achieve total doses of either 25, 125 or 250 kGy. The samples were exposed to gamma rays emitted from high activity sealed cobalt-60 sources loaded into a model 812 irradiator, supplied by Foss Therapy Services, Inc, California, USA. Absorbed dose rates were determined using a model 2060C radiation detection instrument equipped with ion chamber type 20X60-0.18, supplied by Radcal Corporation, California, USA. The model 2060C instrument was calibrated annually to traceable national or international standards. In addition, routine cross-checks of dose rates were performed using Fricke dosimetry, a widely used chemical method, with an acceptable tolerance of within +/- 5% of the Radcal measured values.

2.2. Single crystal growth and X-irradiation

Single crystals of L-his were grown from a saturated aqueous solution by slow evaporation at r.t. Their structure was determined by single crystal X-ray diffraction on a Rigaku Oxford Diffraction Gemini R instrument and was found to be orthorhombic with the space group $P2_12_12_1$ (a = 5.1480(3) Å, b = 7.2330(4) Å, c = 18.8122(11) Å), in agreement with previously published structures (Lehmann et al., 1972; Westhof et al., 1974). X-irradiation of the sample, delivering a total dose of 4 kGy, was performed on a Bruker D5005 X-ray powder diffractometer at a dose rate of 0.72 kGy/h. The diffractometer dose rate was calibrated by irradiation of alanine dosimetry pellets (Bruker) and subsequent EPR analysis using a Bruker e-scan Alanine Dosimeter, which has a specified accuracy of better than 1%.

2.3. Spin trapping

Stock solutions of 2-methyl-2-nitrosopropane (hereafter abbreviated to MNP) at 0.8 M or 1.6 M concentrations were prepared using acetonitrile as a solvent for subsequent 1:20 dilution into the aqueous sample solution. In order to investigate the effects of trace metal contamination on the generation of radicals in solution, spin-trapping experiments were performed by dissolving γ-irradiated L-his powder (≈ 0.27 M) either i) in an aqueous solution of MNP; ii) in water, followed by subsequent addition of MNP (after 3 minutes unless otherwise stated); and iii) in an aqueous solution of ethylenediaminetetraacetic acid (abbreviated to EDTA) at 1 mM concentration, followed by the subsequent addition (after 3 mins) of MNP. Samples were inserted into quartz EPR capillaries (Wilmad-LabGlass 712-SQ-100M) using either a glass Pasteur pipette or a syringe bearing a sterile stainless, chromium-nickel steel needle (Braun Sterican 4665643; 21 G, 120 mm). EPR spectra were recorded as described below, with acquisition of the spectra commencing *ca.* 6 mins after the addition of the spin trapping agent MNP. The free radical 4-hydroxyl-2,2,6,6-tetramethyl-piperidine-1-oxyl (abbreviated TEMPOL) was used to quantify the spin adduct concentrations, as previously reported (Barr et al., 2001). Milli-Q water (18.2 MΩ·cm) buffered at pH 7.2 with a 50 mM phosphate buffer was utilised in all of the spin-trapping experiments.

2.4. EPR experiments and simulations

All EPR experiments were performed on a Bruker EMX spectrometer operating at X-band with a cylindrical cavity (ER 4122 SHQE). Unless otherwise stated experimental parameters were as follows: modulation amplitude 0.1 mT; conversion time and time constant 40.96 ms; number of scans 20. For solid state samples the microwave power was 50.7 µW at 9.7 GHz and in spin-trapping experiments (solution-state) the microwave power was 20 mW at 9.8 GHz. EPR simulations were performed with the Matlab package EasySpin (Stoll and Schweiger, 2006).

2.5. XRF analysis

X-ray fluorescence (XRF) analysis was performed on a Rigaku NEX CG Energy Dispersive(ED)-XRF instrument, using a Cu secondary target.

Additional experimental and simulation details are described in the supplementary material.

3. Results and discussion

The γ-irradiation of L-his powder induces the formation of a persistent radical species as revealed by X-band continuous wave (CW) EPR spectroscopy (Fig. 1a). The same radical species is observed irrespective of the radiation dose or the source of ionising radiation (γ- *vs* X-ray) (see Fig. S1 and S2 in the supplementary material). The EPR spectrum of the L-his single crystal exposed to X-ray radiation is shown in Fig. 1b. The main feature of the single crystal spectrum is an eight line pattern which can be readily attributed to couplings with an α-proton and two inequivalent β-protons of the radical. This observation is consistent with the main radical species being the product of deamination (Scheme 1), as previously reported (Westhof et al., 1974). Weaker additional lines can also be observed in the spectrum (Fig.1b) suggesting the presence of a second, as yet unidentified, radical species. Whilst these features are similar in appearance to satellite lines, which are known to arise from the fraction of radicals containing ^{13}C (I = 1/2), their intensity is inconsistent with the natural abundance of this isotope. The microwave power saturation behaviour of the powder sample (Fig. S3) is also indicative of the presence of one or more additional radical species.

Dissolution of the irradiated L-his powder was undertaken in order to study the reactivity of the radical species in solution, and thereby mirror the treatment of excipients in parenteral formulations reconstituted before injection. Following dissolution of the irradiated powder in water, no EPR signal was detected (Fig. S4e), as expected for a short-lived carbon centred radical (Ambroż et al., 2000; Iravani, 2017). However, when the irradiated powder was dissolved in a spin-trap solution of MNP, a persistent spin-adduct signal was detected with a concentration of *ca*. 0.25 µM (Fig. 2). The observed 18-line EPR spectrum can be assigned to the coupling of the unpaired electron with the nitroxidic nitrogen of the spin-trapping agent, along with one α-proton and two almost equivalent β-protons of the trapped radical species. The hyperfine couplings extracted by simulation of the spectra are in good agreement with previous reports in which the deaminated L-his radical was either formed from γ-irradiation of histidine in the solid (Minegishi et al., 1980) or solution state (Rustgi et al., 1977) followed by spin trapping in solution with MNP (Table 1). This 18-line signal was not obtained by dissolving the non-irradiated L-his powder in a solution containing the spin-trap MNP (Fig. 2a), indicating that the trapped adduct species was indeed formed as a result of the irradiation process. The low-intensity three line background signal evident in Fig. 2a was assigned to the formation of an MNP di-adduct, di-*tert*-butyl nitroxide (DTBN), which commonly occurs in low concentrations with this particular spin-trapping agent (Rustgi et al., 1977).

Table 1. Hyperfine parameters (mT) and *g*-values for the MNP-deaminated L-histidine radical spin-adduct.

		$\boldsymbol{a}_{\mathbf{NO}}^{\mathbf{N}}$	$\boldsymbol{a}_{\boldsymbol{\alpha}}^{\mathbf{H}}$	$\boldsymbol{a}_{\boldsymbol{\beta}\mathbf{1}}^{\mathbf{H}}$	$\boldsymbol{a}_{\boldsymbol{\beta}\mathbf{2}}^{\mathbf{H}}$	$\boldsymbol{g}_{\perp}$	$\boldsymbol{g}_{/\!/}$
This study	$a_{\perp}$	1.442(5)	0.400(5)	0.057(5)	0.048(5)	2.0057(2)	2.0052(2)
	$a_{/\!/}$	1.771(5)	0.333(5)	0.079(5)	0.046(5)		
Previous work (Minegishi et al., 1980)	a_{iso}	1.54	0.41	0.06	0.06	- [a]	- [a]
Previous work (Rustgi et al., 1977)	a_{iso}	1.545	0.392	0.05	0.05	- [a]	- [a]

[a] not determined

If the irradiated powder is firstly dissolved in water and the spin trap MNP added subsequently (i.e., only after the dissolution of the powder), then the spin-adduct signal of the trapped radical is still detectable, at a concentration of *ca*. 0.1 µM (Fig. 3a); due to the lower signal intensity, the modulation depth was increased and as a result the β-proton coupling is not resolved. A second, though less intense, four line signal with a 1:2:2:1 pattern can also be observed in the spectrum. The MNP-histidine spin-adduct has been detected following addition of MNP one hour after dissolution of the irradiated powder in water (Fig. S4). Such a long persistence time of the radical in solution is inconsistent with the expected reactivity of carbon centred radicals, and is in contrast with our inability to directly detect the radical in the absence of a spin-trap which implies a short radical lifetime. It is therefore proposed that, rather than invoking long radical lifetimes in solution to account for the observed spin adduct signals, the deaminated histidine radical must be regenerated in solution after addition of the spin-trap. If for example iron is present in the solution, together with a strong oxidant, Fenton-type reactions may take place leading to the formation of reactive oxygen species (ROS) (Neyens and Baeyens, 2003). Such species could facilitate the regeneration of the deaminated histidine radical which is readily and subsequently trapped by MNP at some prolonged time-interval following dissolution of L-his.

To test this hypothesis, irradiated L-his powder was dissolved in an aqueous solution containing the chelating agent EDTA (1 mM), and MNP was subsequently added to this solution 3 mins after dissolution of L-his. As shown in Fig. 3b, the EPR signal from the L-his radical-adduct could not be detected when the chelating agent was present in the solution. In our initial experiments, a syringe fitted with a sterile metal needle was used to transfer the solution to the quartz capillary for EPR measurement. However, when a glass pipette was used instead to transfer the sample solution into the EPR quartz capillary tube, no spin-adducts were detected even in the absence of EDTA (Fig. 3c). An X-ray fluorescence analysis of the metal needles showed the presence of large amounts of iron, together with chromium, manganese, nickel and trace levels of other metals (Fig. S6). It therefore appears that the deaminated radical trapped after dissolution in water and subsequent addition of MNP is the result of a Fenton-type reaction catalysed by traces of the metals contained in the syringe needle, which takes place as soon as the needle comes in contact with the sample solution. Furthermore, such behaviour appears to be characteristic of L-histidine, as other excipients such as D-mannitol did not show any radical regeneration properties (Fig. S7). This result is of great significance for the use of irradiation sterilization of excipients in parenteral formulations, for which the reconstitution process or drug delivery might involve use of similar needles providing sufficient trace metals for radical regeneration.

According to these considerations, the four line EPR spectrum of the previously unidentified radical species can be attributed to the formation of the MNP-OH spin-adduct (Fig. S5), further supporting the assertion that Fenton-type reactions are operative. Additionally, the concentration of the MNP-histidine radical-adduct formed from direct dissolution of the irradiated powder in the spin trap solution was found to be four times higher (*ca.* 1 μM) when the glass pipette was used in place of the syringe with metal needle (*ca.* 0.25 μM). The proposed Fenton-type reactions could also explain why the concentration of the MNP-His spin-adduct was found to be significantly lower when using the syringe and needle for sample transfer, as the reactions of the ROS produced might compete with direct formation of the spin-adduct from the L-his deaminated in the solid state.

As mentioned earlier, the presence of strong oxidants is required for the Fenton chemistry to occur. Such oxidants can easily form as a result of the irradiation process. In fact, the primary effect of exposing L-his to ionising radiations is the ejection of an electron from the molecule itself (Symons, 1995) (Scheme 2). Thus, in addition to the formation of a histidine radical, in the presence of air, the ejected electron can also combine with molecular oxygen leading to the formation of a superoxide radical. This reactive oxygen species can in turn lead to the generation of other ROS such as hydrogen peroxide and histidine hydroperoxides. Irradiation in solution is known to produce amino acid hydroperoxides from which spin adducts can be trapped on addition of Fe^{2+} (Davies et al., 1995; Gebicki, S., Gebicki, J.M., 1993), but to our knowledge generation of these species by irradiation in the solid state and survival into solution has not previously been observed. All these species can be responsible for initiating the observed Fenton chemistry in solution and the consequent production of additional ROS. The well-known scavenging properties of L-his towards ROS (Foote and Clennan, 1995; Pazos et al., 2006; Wade and Tucker, 1998; Zs.-Nagy and Floyd, 1984) suggest that these reactive species are quenched by histidine in solution, hence further generating deamination radicals which are readily trapped by MNP.

4. Conclusions

The effects of irradiation sterilization on the parenteral excipient L-his has been analysed by CW EPR spectroscopy and spin-trapping. Whilst the identity of the irradiation induced deamination radical formed has been confirmed previously through both direct analysis of the irradiated solid and spin-trapping experiments, in this work we have further explored the fate of the irradiation products in solution. As expected upon dissolution of the irradiated powder in a physiological solution, the radical species were found to have a short lifetime; however, spin-trapping experiments show not only the formation of C-centred radical adducts, but also the regeneration of radical species long after the initial dissolution of the irradiated material. Fenton-type chemistry involving strong oxidants generated during the irradiation process, and catalysed by trace metals from a standard sterile syringe needle, was implicated in this process.

Knowing the behaviour of the reactive degradation products in solution is essential when dealing with excipients intended for parenteral formulations. Avoiding the regeneration of the radicals in solution is necessary in order to eliminate the potential for radical-induced degradation of other drug components, such as APIs in particular, in a complete pharmaceutical formulation. Unintentional injection of free radical containing solutions into patients could also have direct toxicological implications. Whilst a thorough analysis of each irradiation sterilized product remains necessary, assessing the effects of γ-irradiation on single drug ingredients is an essential first step towards the analysis of multi-component systems. We have shown that not only the degradants formed directly by the irradiation procedure, but also the subsequent products of potentially complex solution mechanisms, must be taken into account.

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Figure Captions

Fig. 1. a) X-band CW EPR spectrum of L-histidine (L-his) powder after γ-irradiation at r.t. with a total dose of 25 kGy. b) X-band CW EPR spectrum of L-his single crystal with the magnetic field aligned parallel to the *c*-axis after X-irradiation at r.t. (black) and EasySpin (Stoll and Schweiger, 2006) simulation (dashed red). The EPR parameters used to record the powder & single crystal spectra respectively were: a) time constant 81.92 ms; number of points 1024; number of scans 4 and b) time constant 40.96 ms; number of points 2048; number of scans 20, with other parameters as in section 2.4.

Fig. 2. X-band CW EPR spectra of a) non-irradiated L-his powder dissolved in a spin-trap solution of MNP (80 mM), b) 250 kGy γ-irradiated L-his powder dissolved in a spin-trap solution of MNP (80 mM), c) simulated EPR spectrum (obtained using EasySpin (Stoll and Schweiger, 2006)) for a combination of the MNP-his adduct and DTBN, and d) simulation of DTBN only. The EPR parameters used to record the spin-adduct spectra were as detailed in section 2.4 with the exception of the lower modulation amplitude of 0.01 mT; the number of points was 4096.

Fig. 3. X-band CW EPR spectra of L-his powder dissolved in water with a) MNP (final conc. 20 mM) added 3 minutes after dissolution and transferred using a syringe with metal needle; b) aqueous EDTA (1 mM) followed by processing as described in a); c) same as a) but transferred by using a glass Pasteur pipette. In a) the low-intensity four line EPR spectrum with a 1:2:2:1 pattern, indicated by ▼, was attributed to the spin-adduct MNP-OH (see Fig. S5 and Table S1). The EPR parameters used to record the spin-adduct spectra were as detailed in section 2.4, with number of points 1024.

Scheme 1. Radiolytic deamination of L-his.

Scheme 2. Irradiation of L-his powder with formation of strong oxidants (labelled in blue) involved in the regeneration of L-his radicals in solution. The ROS produced from the Fenton reaction (in red) are scavenged by L-his, with the consequent formation of L-his radicals readily trapped by MNP to form species detected by EPR (magenta).